Safety evaluation of the food enzyme thermolysin from the non-genetically modified Anoxybacillus caldiproteolyticus strain AE-TP

EFSA Panel on Food Contact Materials, Enzymes and Processing Aids (CEP)

Published in:
EFSA Journal

Link to article, DOI:
10.2903/j.efsa.2024.8634

Publication date:
2024

Document Version
Publisher's PDF, also known as Version of record

Adopted: 1 February 2024

DOI: 10.2903/j.efsa.2024.8634

efsa JOURNAL

SCIENTIFIC OPINION

Safety evaluation of the food enzyme thermolysin from the non-genetically modified *Anoxybacillus caldiproteolyticus* strain AE-TP

EFSA Panel on Food Contact Materials, Enzymes and Processing Aids (CEP) | Claude Lambré | José Manuel Barat Baviera | Claudia Bolognesi | Pier Sandro Cocconcelli | Riccardo Crebelli | David Michael Gott | Konrad Grob | Evgenia Lampi | Marcel Mengelers | Alicja Mortensen | Gilles Rivière | Inger-Lise Steffensen | Christina Tlustos | Henk Van Loveren | Laurence Vernis | Holger Zorn | Lieve Herman | Yrjö Roos | Magdalena Andryszkiewicz | Yi Liu | Simone Lunardi | Elsa Nielsen | Karin Nørby | Andrew Chesson

Correspondence: fip@efsa.europa.eu

Abstract

The food enzyme thermolysin (EC. 3.4.24.27) is produced with the non-genetically modified *Anoxybacillus caldiproteolyticus* strain AE-TP by Amano Enzyme Inc. The food enzyme is free from viable cells of the production organism. It is intended to be used in eight food manufacturing processes. Dietary exposure was estimated to be up to 0.973 mg TOS/kg body weight (bw) per day in European populations. Genotoxicity tests did not indicate a safety concern. The systemic toxicity was assessed by means of a repeated dose 90-day oral toxicity study in rats. The Panel identified a no observed adverse effect level of 700 mg TOS/kg bw per day, the mid-dose tested, which, when compared with the estimated dietary exposure, resulted in a margin of exposure of at least 719. A search for the similarity of the amino acid sequence of the food enzyme to known allergens was made and no matches were found. The Panel considered that the risk of allergic reactions by dietary exposure cannot be excluded, but the likelihood is low. Based on the data provided, the Panel concluded that this food enzyme does not give rise to safety concerns under the intended conditions of use.

KEYWORDS

Anoxybacillus caldiproteolyticus, EC 3.4.24.27, food enzyme, thermoase, thermolysin

CONTENTS

Abstract 1
1. Introduction 3
   1.1. Background and Terms of Reference as provided by the requestor 3
      1.1.1. Background as provided by the European Commission 3
      1.1.2. Terms of Reference 3
   1.2. Interpretation of the Terms of Reference 4
2. Data and methodologies 4
   2.1. Data 4
   2.2. Methodologies 4
3. Assessment 4
   3.1. Source of the food enzyme 4
   3.2. Production of the food enzyme 5
   3.3. Characteristics of the food enzyme 5
      3.3.1. Properties of the food enzyme 5
      3.3.2. Chemical parameters 5
      3.3.3. Purity 6
      3.3.4. Viable cells of the production strain 6
   3.4. Toxicological data 6
      3.4.1. Genotoxicity 6
         3.4.1.1. Bacterial reverse mutation test 6
         3.4.1.2. In vitro mammalian cell micronucleus test 7
      3.4.2. Repeated dose 90-day oral toxicity study in rodents 7
      3.4.3. Allergenicity 8
   3.5. Dietary exposure 8
      3.5.1. Intended use of the food enzyme 8
      3.5.2. Dietary exposure estimation 10
      3.5.3. Uncertainty analysis 10
   3.6. Margin of exposure 11
4. Conclusions 11
5. Documentation as provided to EFSA 11
Abbreviations 11
Conflict of Interest 11
Requestor 11
Question Number 11
Copyright for non-EFSA Content 11
Panel Members 12
Note 12
References 12
Appendix A 13
Appendix B 14

1 | INTRODUCTION

Article 3 of the Regulation (EC) No 1332/2008[1] provides definition for 'food enzyme' and 'food enzyme preparation'.

'Food enzyme' means a product obtained from plants, animals or micro-organisms or products thereof including a product obtained by a fermentation process using micro-organisms: (i) containing one or more enzymes capable of catalysing a specific biochemical reaction; and (ii) added to food for a technological purpose at any stage of the manufacturing, processing, preparation, treatment, packaging, transport or storage of foods.

'Food enzyme preparation' means a formulation consisting of one or more food enzymes in which substances such as food additives and/or other food ingredients are incorporated to facilitate their storage, sale, standardisation, dilution or dissolution.

Before January 2009, food enzymes other than those used as food additives were not regulated or were regulated as processing aids under the legislation of the Member States. On 20 January 2009, Regulation (EC) No 1332/2008 on food enzymes came into force. This Regulation applies to enzymes that are added to food to perform a technological function in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food, including enzymes used as processing aids. Regulation (EC) No 1331/2008[2] established the European Union (EU) procedures for the safety assessment and the authorisation procedure of food additives, food enzymes and food flavourings. The use of a food enzyme shall be authorised only if it is demonstrated that:

- it does not pose a safety concern to the health of the consumer at the level of use proposed;
- there is a reasonable technological need;
- its use does not mislead the consumer.

All food enzymes currently on the European Union market and intended to remain on that market, as well as all new food enzymes, shall be subjected to a safety evaluation by the European Food Safety Authority (EFSA) and approval via an EU Community list.

1.1 | Background and Terms of Reference as provided by the requestor

1.1.1 | Background as provided by the European Commission

Only food enzymes included in the European Union (EU) Community list may be placed on the market as such and used in foods, in accordance with the specifications and conditions of use provided for in Article 7(2) of Regulation (EC) No 1332/2008[1] on food enzymes.

Five applications have been introduced by the companies 'Amano Enzyme Inc.' for the authorisation of the food enzymes cyclomaltodextrin glucanotransferase from *Geobacillus stearothermophilus* (strain AE-KCGT), cyclomaltodextrin glucanotransferase from *Paenibacillus macerans* (strain AE-CGT) and thermolysin from *Geobacillus stearothermophilus* (strain AE-TP), 'Sanyo Fine Co., Ltd.' for the authorisation of the food enzyme phospholipase A2 from porcine pancreas and 'Nagase (Europa) GmbH' for the authorisation of the food enzyme beta-amylase from soybean (*Glycine max*).

Following the requirements of Article 12.1 of Commission Regulation (EU) No 234/2011[3] implementing Regulation (EC) No 1331/2008[2], the Commission has verified that the five applications fall within the scope of the food enzyme Regulation and contains all the elements required under Chapter II of that Regulation.

1.1.2 | Terms of Reference

The European Commission (EC) requests the European Food Safety Authority (EFSA) to carry out the safety assessments on the food enzymes cyclomaltodextrin glucanotransferase from *Geobacillus stearothermophilus* (strain AE-KCGT), cyclomaltodextrin glucanotransferase from *Paenibacillus macerans* (strain AE-CGT), thermolysin from *Geobacillus stearothermophilus* (strain AE-TP), phospholipase A2 from porcine pancreas and beta-amylase from soybean (*Glycine max*) in accordance with Article 17.3 of Regulation (EC) No 1332/2008[1] on food enzymes.

[1]Regulation (EC) No. 1332/2008 of the European Parliament and of the Council of 16 December 2008 on Food Enzymes and Amending Council Directive 83/417/EEC, Council Regulation (EC) No. 1493/1999, Directive 2000/13/EC, Council Directive 2001/112/EC and Regulation (EC) No 258/97. OJ L 354, 31.12.2008, pp. 7–15.

[2]Regulation (EC) No. 1331/2008 of the European Parliament and of the Council of 16 December 2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 354, 31.12.2008, pp. 1–6.

[3]Commission Regulation (EU) No 234/2011 of 10 March 2011 implementing Regulation (EC) No 1331/2008 of the European Parliament and of the Council establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 64, 11.03.2011, pp. 15–24.

1.2 | Interpretation of the Terms of Reference

The present scientific opinion addresses the European Commission's request to carry out the safety assessment of the food enzyme thermolysin from a non-genetically modified *G. stearothermophilus* strain AE-TP.

Recent data identified the production microorganism as *Anoxybacillus caldiproteolyticus* (Section 3.1). Therefore, this name will be used in this opinion instead of *Geobacillus stearothermophilus*.

2 | DATA AND METHODOLOGIES

2.1 | Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme thermolysin from the non-genetically modified *A. caldiproteolyticus* strain AE-TP.

Additional information was requested from the applicant during the assessment process on 13 December 2021 and 21 September 2022, and received on 27 April 2022 and 19 September 2023, respectively (see 'Documentation as provided to EFSA').

2.2 | Methodologies

The assessment was conducted in line with the principles described in the EFSA 'Guidance on transparency in the scientific aspects of risk assessment' (EFSA, 2009) and following the relevant guidance documents of the EFSA Scientific Committee.

The 'Guidance on the submission of a dossier on food enzymes for safety evaluation' (EFSA CEF Panel, 2009) as well as the 'Statement on characterisation of microorganisms used for the production of food enzymes' (EFSA CEP Panel, 2019) have been followed for the evaluation of the application. Additional information was requested in accordance with the updated 'Scientific Guidance for the submission of dossiers on food enzymes' (EFSA CEP Panel, 2021) and the guidance on the 'Food manufacturing processes and technical data used in the exposure assessment of food enzymes' (EFSA CEP Panel, 2023).

3 | ASSESSMENT

IUBMB nomenclature	Thermolysin
Systematic name	–
Synonyms	*Bacillus thermoproteolyticus* neutral proteinase; thermoase
IUBMB no	EC. 3.4.24.27
CAS no	37246-64-3
EINECS no	253-424-5

Thermolysins catalyse the hydrolysis of peptide bonds of proteins with broad specificity, but preferentially, between leucine (Leu) and phenylalanine (Phe), releasing peptides and amino acids.

The enzyme under assessment is intended to be used in eight food manufacturing processes as described in the EFSA guidance (EFSA CEP Panel, 2023): (1) processing of eggs and egg products; processing of dairy products for the production of (2) flavouring preparations and (3) modified milk proteins; processing of meat and fish products for the production of (4) modified meat and fish products and (5) protein hydrolysates; processing of plant- and fungal-derived products for the production of (6) plant-based analogues of milk and milk products and (7) protein hydrolysates and (8) processing of yeast and yeast products.

3.1 | Source of the food enzyme

The thermolysin is produced with the non-genetically modified bacterium *Anoxybacillus caldiproteolyticus* strain AE-TP, which is deposited at the National Institute of Technology and Evaluation (NITE) Biological Resurce Centre (Japan), with the deposit number [redacted][4] The production strain was identified as *A. caldiproteolyticus* by whole genome sequencing (WGS), [redacted][5]

[4]Technical dossier/Additional information September 2023/Annex 2.

[5]Technical dossier/Additional information September 2023/Annex 1.

The production strain *A. caldiproteolyticus* AE-TP was derived from [redacted] isolate by [redacted].

A search for antimicrobial resistance (AMR) genes was performed on the genome of the production strain using two regularly updated databases with a minimum identity of 80% and minimum coverage of 70%. No genes of concern were found.[6]

3.2 | Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004,[7] with food safety procedures based on Hazard Analysis and Critical Control Points, and in accordance with current Good Manufacturing Practice.[8]

The production strain is grown as a pure culture using a typical industrial medium in a submerged, fed-batch fermentation system with conventional process controls in place. After completion of the fermentation, the solid biomass is removed from the fermentation broth by filtration. The filtrate containing the enzyme is then further purified and concentrated, including an ultrafiltration step in which enzyme protein is retained, while most of the low molecular mass material passes the filtration membrane and is discarded.[9] The applicant provided information on the identity of the substances used to control the fermentation and in the subsequent downstream processing of the food enzyme.[10]

The Panel considered that sufficient information has been provided on the manufacturing process and the quality assurance system implemented by the applicant to exclude issues of concern.

3.3 | Characteristics of the food enzyme

3.3.1 | Properties of the food enzyme

The thermolysin is a single polypeptide chain of [redacted] amino acids.[11] The molecular mass of the mature protein was calculated from the amino acid sequence to be [redacted] kDa. The food enzyme was analysed by size exclusion chromatography. The chromatograms of the three food enzyme batches for commercialisation showed a consistent pattern.[12] No other relevant enzymatic activities were reported.[13]

The determination of thermolysin activity is based on the hydrolysis of casein (reaction conditions: pH 7.0, 37°C, 60 min), spectrophotometrically measuring the release of amino acids that react with Folin's test solution at 660 nm. The thermolysin activity is expressed in protein digestive activity units (U)/g. One U is the amount of enzyme that produces an increase in absorbance equivalent to that produced by 1 µg of tyrosine per minute under the conditions of the assay.[14]

The food enzyme has a temperature optimum around 65°C (pH 7.2) and a pH optimum around pH 7.5 (35°C). Thermostability was tested after a pre-incubation of the food enzyme for 60 min at different temperatures (pH 7.0). The enzyme activity decreased above 45°C, showing no residual activity above 80°C.[15]

3.3.2 | Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three batches used for commercialisation and one batch used in the toxicological studies (Table 1).[16] The mean total organic solids (TOS) of the three batches was 42.2% and the mean enzyme activity/TOS ratio was 11,178 U/mg TOS.

[6]Technical dossier/Additional information September 2023/Annex 1.

[7]Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of food additives. OJ L 226, 25.6.2004, pp. 3–21.

[8]Technical dossier/p. 40/Annex 4.

[9]Technical dossier/p. 40–48/Annex 5.

[10]Technical dossier/Annex 6.

[11]Technical dossier/p. 30–31.

[12]Technical dossier/p. 29.

[13]Technical dossier/p. 32.

[14]Technical dossier/p. 31/Annex 2.

[15]Technical dossier/p. 32–33.

[16]Technical dossier/p. 28/Annexes: 1 and 3.

TABLE 1 Composition of the food enzyme.

Parameters	Unit	Batches			
		1	2	3	4[b]
Thermolysin activity	U/g batch[a]	4,500,000	4,710,000	4,940,000	1,230,000
Protein	%	41.1	38.0	40.7	12.2
Ash	%	54.8	56.0	55.1	4.7
Water	%	2.7	2.5	2.3	82.3
Total organic solids (TOS)[c]	%	42.5	41.5	42.6	13.0
Activity/TOS ratio	U/mg TOS	10,588	11,349	11,596	9462

[a]UNIT: U/g (see Section 3.3.1).
[b]Batch used for the toxicological studies.
[c]TOS calculated as 100% – % water – % ash.

3.3.3 | Purity

The lead content in the three commercial batches and in the batch used for toxicological studies was below 0.06 mg/kg,[17,18] which complies with the specification for lead as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006). The level of mercury was below the limit of detection (LoD) of the employed method. For arsenic and cadmium, the mean concentrations determined in the commercial batches were 0.31 mg/kg and 0.18 mg/kg, respectively.[19,20] The Panel considered these concentrations as not of safety concern.

The food enzyme preparation complies with the microbiological criteria for total coliforms, *Escherichia coli* and *Salmonella*, as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006).[21] No antimicrobial activity was detected in any of the tested batches.[22]

The Panel considered that the information provided on the purity of the food enzyme was sufficient.

3.3.4 | Viable cells of the production strain

The absence of viable cells of the production strain in the food enzyme was demonstrated in three independent batches analysed in triplicate. [redacted]. A positive control was included.[23]

3.4 | Toxicological data

A battery of toxicological tests has been provided, including a bacterial reverse mutation test (Ames test), an in vitro mammalian cell micronucleus test and a repeated dose 90-day oral toxicity study in rats.[24]

The batch 4 (Table 1) used in these studies was prepared without the inorganic material used to stabilise the batches used for commercialisation. It had a lower activity/TOS value and was considered suitable as a test item.

3.4.1 | Genotoxicity

3.4.1.1 | *Bacterial reverse mutation test*

A bacterial reverse mutation test (Ames test) was performed according to the Japanese 'Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use' (2012), the Organisation for Economic Co-operation and Development (OECD) Test Guideline 471 (OECD, 2020) and following Good Laboratory Practice (GLP).[25]

Four strains of *Salmonella* Typhimurium (TA98, TA100, TA1535 and TA1537) and *Escherichia coli* WP2uvrA(pKM101) were used with or without metabolic activation (S9-mix), applying the pre-incubation method. A range-finding test was carried

[17]Technical dossier/p. 30/Annexes 1 and 3 and Additional information September 2023/Annex 3.
[18]LoD: Pb = 0.005 mg/kg.
[19]Technical dossier/p. 30/Annexes 1 and 3.
[20]LoDs: Cd = 0.001 mg/kg; As = 0.002 mg/kg; Hg = 0.001 mg/kg.
[21]Technical dossier/p. 30/Annexes: 1 and 3 and Additional information September 2023/Annex 3.
[22]Technical dossier/p. 30/Annexes: 1 and 3.
[23]Technical dossier/Additional information September 2023/Annexes 7–1 and 7–2.
[24]Technical dossier/Additional information September 2023/Annexes 3–6.
[25]Technical dossier/Additional information, September 2023/Annex 4.

out in duplicate, using five concentrations of the food enzyme ranging from 19.5 to 5000 µg TOS/plate. Precipitates were observed at 5000 µg TOS/plate with or without S9-mix. No cytotoxicity was seen at any concentration tested up to 5000 µg TOS/mL with and without S9-mix.

Based on these results, two main experiments were carried out in triplicate, using five concentrations of the food enzyme of 313, 625, 1250, 2500 and 5000 µg TOS/plate. Precipitates were observed at 5000 µg TOS/plate with or without S9-mix. No cytotoxicity was observed at any concentration of the test substance. Upon treatment with the food enzyme, there was no biologically relevant increase in the number of revertant colonies above the control values in any strain tested, with or without S9-mix.

The Panel concluded that the food enzyme thermolysin did not induce gene mutations under the test conditions applied in this study.

3.4.1.2 | *In vitro mammalian cell micronucleus test*

The in vitro mammalian cell micronucleus test was carried out according to the OECD Test Guideline 487 (OECD, 2016) and following GLP.[26] An experiment was performed with duplicate cultures of the human lymphoblastoid TK6 cell line. The cell cultures were treated with the food enzyme with or without metabolic activation (S9-mix).

In the range-finding test, cells were exposed to the food enzyme at ten concentrations ranging from 9.77 to 5000 µg TOS/mL in a short-term treatment (4 h exposure and 20 h recovery period), either with or without S9-mix, and in a long-term treatment (24 h exposure without recovery period) without S9-mix. Cytotoxicity of ≥ 50% (the cell growth inhibition rate) was observed at ≥ 1250 µg TOS/mL in the short-term treatment without S9-mix and at ≥ 2500 µg TOS/mL in the short-term treatment with S9-mix and in the long-term treatment without S9-mix. The 50% cell-growth inhibition concentration (IC_{50}) was at 857 µg TOS/mL in the short-term treatment without S9-mix, 2020 µg TOS/mL in the short-term treatment with S9-mix and 2160 µg TOS/mL in the long-term treatment without S9-mix, respectively.

Based on these results, in the main experiment, cells were exposed to the food enzyme and scored for the frequency of cells with micronuclei at concentrations of 500, 650 and 800 µg TOS/mL in a short-term treatment without S9-mix, at concentrations of 600, 900, 1200 and 1500 µg TOS/mL in a short-term treatment with S9-mix and at concentrations of 1200, 1500 and 1800 µg TOS/mL in a long-term treatment without S9-mix.

A 50% cell growth inhibition was seen either at the highest concentration tested in the short-term with and/or without S9-mix or in the long-term treatment.

The frequency of micronucleated cells was not statistically significantly different to the negative controls at any concentrations tested.

The Panel concluded that the food enzyme thermolysin did not induce an increase in the frequency of micronucleated cells under the test conditions applied in this study.

3.4.2 | Repeated dose 90-day oral toxicity study in rodents

The repeated dose 90-day oral toxicity study followed the OECD Test Guideline 408 (OECD, 2018) and GLP.[27]

Groups of 10 male and 10 female Sprague–Dawley (Crl:CD(SD)) rats received the food enzyme in doses of 350, 700 or 1400 mg TOS/kg bw per day by gavage. Controls received the vehicle (water for injection).

No mortality was observed.

The feed consumption was statistically significantly increased on Day 1 of administration in low- and mid-dose males (+8% and +8%, respectively). The Panel considered the change as not toxicologically relevant, as it was only recorded at a single time interval, it was only observed in one sex, there was no dose–response relationship and there was no statistically significant change in the final feed consumption, body weight and body weight gain.

In the functional observations, a statistically significant decrease in the rearing count was observed in high-dose males (−50%) in week 2. The Panel considered the change as not toxicologically relevant, as it was only recorded at a single time interval and it was only observed in one sex.

Clinical chemistry investigations revealed a statistically significant decrease in sodium in low-, mid- and high-dose males (−1%, −0.7% and −1%, respectively), an increase in blood urea nitrogen (BUN, +24%) and in urea calculated from BUN (+19%) in high-dose females. The Panel considered the changes as not toxicologically relevant, as they were only observed in one sex, there was no dose–response relationship (sodium) and there were no histopathological changes in the kidneys.

Statistically significant changes in hormone levels included a decrease in tri-iodothyronine (T3) in high-dose males (−10%) and a decrease in thyroxine (T4) in mid-dose males (−14%). The Panel considered the changes as not toxicologically relevant, as they were only observed in one sex (both parameters), there was no dose–response relationship (T4), there were no histopathological changes in thyroid gland and there were no changes in other relevant parameters (thyroid-stimulating hormone level).

[26]Technical dossier/Additional information, September 2023/Annex 5.

[27]Technical dossier/Additional information, September 2023/Annex 6.

The urinalysis revealed a statistically significant increase in the urine volume in high-dose males (+42%). The Panel considered the change as not toxicologically relevant, as it was only observed in one sex and there were no histopathological changes in the kidneys.

Statistically significant changes in organ weights detected were an increase in the absolute pituitary weight in low- and high-dose males (+14% and + 23%, respectively), an increase in the relative pituitary weight (+13%) and in the absolute heart weight (+11%) in high-dose males, an increase in the absolute lung weight in mid-dose males (+18%) and an increase in the absolute seminal vesicle weight in low-dose males (+18%). The Panel considered the changes as not toxicologically relevant, as they were only observed in one sex (the absolute and relative pituitary weight, the absolute heart and lung weights), there was no dose–response relationship (absolute pituitary, lung and seminal vesicle weights) and there were no histopathological changes in pituitary gland, heart and seminal vesicles.

The macroscopic examination of the stomach revealed dark red foci in the glandular stomach in the control and low-dose males (1/10 and 1/10, respectively) and in low-dose females (2/10 vs 0/10 in the control). These changes were consistent with the microscopic observations of minimal erosion/ulceration in the glandular stomach in these animals and additionally in one high-dose females. Furthermore, the microscopic examination of the stomach revealed minimal hyperplasia of the squamous cells in the limiting ridge in mid- and high-dose males (1/10 and 8/10 vs 0/10 in the control) and in mid- and high-dose females (1/10 and 6/10 vs 0/10 in the control). The Panel considered the changes in the stomach as test item-related. The minimal severity indicated absence of adversity, which was supported by absence of toxicologically significant changes in clinical pathology. On the other hand, the Panel noted a significant increase in the incidence of hyperplasia of the squamous cells in the limiting ridge at the high dose.

No other statistically significant or biologically relevant differences to controls were reported.

The Panel identified a no observed adverse effect level (NOAEL) of 700 mg TOS/kg bw per day (the mid-dose), based on the increase in the incidence of hyperplasia of the squamous cells in the limiting ridge of the stomach observed in both sexes at the high dose.

3.4.3 | Allergenicity

The allergenicity assessment considers only the food enzyme and not any carrier or other excipient that may be used in the final formulation.

The potential allergenicity of the thermolysin produced with *A. caldiproteolyticus* strain AE-TP was assessed by comparing its amino acid sequence with those of known allergens according to the 'Scientific opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed of the Scientific Panel on Genetically Modified Organisms' (EFSA GMO Panel, 2010). Using higher than 35% identity in a sliding window of 80 amino acids as the criterion, no match was found.[28]

No information was available on oral and respiratory sensitisation or elicitation reactions of this thermolysin. No allergic reactions to thermolysin have been reported in the literature.

[redacted], products that may cause allergies (listed in the Regulation (EU) No 1169/2011[29]), are used as raw materials. In addition, [redacted], known sources of allergens, are present in the media fed to the microorganisms. However, during the fermentation process, these products will be degraded and utilised by the microorganisms for cell growth, cell maintenance and production of enzyme protein. In addition, the microbial biomass and fermentation solids are removed. Taking into account the fermentation process and downstream processing, the Panel considered that potentially allergenic residues from these sources are not expected to be present.

The Panel considered that the risk of allergic reactions upon dietary exposure to this food enzyme cannot be excluded but the likelihood is low.

3.5 | Dietary exposure

3.5.1 | Intended use of the food enzyme

The food enzyme is intended to be used in eight food manufacturing processes at the recommended use levels summarised in Table 2.

[28]Technical dossier/p. 65–66/Annex 8.

[29]Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004.

TABLE 2 Intended uses and recommended use levels of the food enzyme as provided by the applicant.[30]

Food manufacturing process[a]	Raw material (RM)	Maximum recommended use level (mg TOS/kg RM)[b]	
Processing of eggs and egg products	Whole egg/egg yolk/egg white	**1.3**	
Processing of dairy products			
• Production of flavouring preparations from dairy products	Cheese, curd[31]	**1.3**	
• Production of modified milk proteins[32]	Milk proteins	**375.4**	Infant formulae
		62.6	Other uses
Processing of meat and fish products			
• Production of modified meat and fish products	Meat and fish	**1.3**	
• Production of protein hydrolysates from meat and fish proteins[32]	Meat and fish proteins	**62.6**	
Processing of plant- and fungal-derived products			
• Production of plant-based analogues of milk and milk products[33]	Plant materials	**0.3**	
• Production of protein hydrolysates from plants and fungi[32]	Plant proteins	**375.4**	Infant formulae
		62.6	Other uses
Processing of yeast and yeast products	Dried yeast[34]	**1.3**	

Abbreviation: TOS, total organic solids.

[a]The name has been harmonised by EFSA according to the 'Food manufacturing processes and technical data used in the exposure assessment of food enzymes' (EFSA CEP Panel, 2023).

[b]The numbers in bold represent the maximum recommended use levels which were used for calculation.

In the processing of eggs and egg products, the food enzyme is added to treat the whole egg or egg white and yolk.[35] The hydrolysis by thermolysin enhances the sensory properties of the final products.[36] The food enzyme–TOS remains in these enzyme-modified egg products that are used as ingredients in a variety of final foods (e.g. prepared foods, mayonnaise, dressings, sauces, pastries).

In the production of flavouring preparations from dairy products, the food enzyme is added to curd to hydrolyse proteins during the incubation step,[37] giving a savoury flavour to the resulting enzyme-modified cheeses (EMCs).[38] The food enzyme–TOS remains in the EMC, which is used as an ingredient in a variety of final foods (e.g. processed cheese, soups, snacks, dressings, sauces).

In the production of protein hydrolysates, the food enzyme is added to plant and animal protein-rich materials (e.g. whey protein, caseins, collagen, corn protein, soybean protein).[39] The hydrolysis by thermolysin improves the yield. The food enzyme–TOS remains in the final protein hydrolysates that are used as ingredients in a variety of final foods, including infant formulae and follow-on formulae.

In the production of modified meat and fish products, the food enzyme is added to the broth to obtain meat and fish extracts[40] or to meat and fish flesh during the mincing phase to obtain products like steaks or hamburgers.[41] The hydrolysis by thermolysin reduces viscosity and enhances the flavour of the extracts.[42] When used for tenderising purposes, the enzymatic treatment softens meat in steaks or hamburgers.[43] The food enzyme–TOS remains in the final foods.

In the production of plant-based analogues of milk and milk products, the food enzyme is added to plant materials (e.g. soybean, oat flour, almond flour and rice flour) to enrich the flavour of the final foods.[44] The food enzyme–TOS remains in the final foods (e.g. plant-based beverages and their fermented products).

In the processing of yeast and yeast products, the food enzyme is added to the yeast culture during the lysis step or directly to yeast extract.[45] The enzyme is used to enrich the savoury taste of the yeast products that are used (in paste or powder form) as ingredients in a wide range of foods. The food enzyme–TOS remains in the final food products.

[30]Additional information September 23/p. 1–2
[31]Additional information April 22/Answer 7.
[32]Additional information April 22/Answer 9.
[33]Additional information April 22/Answer 12.
[34]Additional information April 22/Answer 10.
[35]Technical dossier/p. 52.
[36]Technical dossier/p. 77.
[37]Technical dossier/p. 51.
[38]Technical dossier/p. 75.
[39]Technical dossier/p. 57.
[41]Technical dossier/pp. 54–56.
[40]Technical dossier/p. 53.
[42]Technical dossier/p. 79.
[43]Technical dossier/p. 81.
[44]Technical dossier/Additional information April 22/Answer to question 12.
[45]Technical dossier/p. 58.

Based on data provided on thermostability (see Section 3.3.1) and the downstream processing step applied in the food manufacturing processes, it is expected that the food enzyme is inactivated in all food manufacturing processes listed in Table 2.

3.5.2 | Dietary exposure estimation

Chronic exposure to the food enzyme–TOS was calculated by combining the maximum recommended use level with individual consumption data (EFSA CEP Panel, 2021). The estimation involved selection of relevant food categories and application of technical conversion factors (EFSA CEP Panel, 2023). Exposure from all FoodEx categories was subsequently summed up, averaged over the total survey period (days) and normalised for body weight. This was done for all individuals across all surveys, resulting in distributions of individual average exposure. Based on these distributions, the mean and 95th percentile exposures were calculated per survey for the total population and per age class. Surveys with only 1 day per subject were excluded and high-level exposure/intake was calculated for only those population groups in which the sample size was sufficiently large to allow calculation of the 95th percentile (EFSA, 2011).

Table 3 provides an overview of the derived exposure estimates across all surveys. Detailed mean and 95th percentile exposure to the food enzyme–TOS per age class, country and survey, as well as contribution from each FoodEx category to the total dietary exposure are reported in Appendix A – Tables 1 and 2. For the present assessment, food consumption data were available from 48 dietary surveys (covering infants, toddlers, children, adolescents, adults and the elderly), carried out in 26 European countries (Appendix B). The highest dietary exposure was estimated to be 0.973 mg TOS/kg bw per day in infants at the 95th percentile.

TABLE 3 Summary of the estimated dietary exposure to food enzyme–TOS in six population groups.

	Estimated exposure (mg TOS/kg body weight per day)					
Population group	**Infants**	**Toddlers**	**Children**	**Adolescents**	**Adults**	**The elderly**
Age range	3–11 months	12–35 months	3–9 years	10–17 years	18–64 years	≥ 65 years
Min–max mean (number of surveys)	0.010–0.290 (12)	0.013–0.155 (15)	0.009–0.017 (19)	0.002–0.010 (21)	0.002–0.008 (22)	0.001–0.006 (23)
Min–max 95th percentile (number of surveys)	0.024–0.973 (11)	0.034–0.448 (14)	0.020–0.046 (19)	0.006–0.034 (20)	0.005–0.027 (22)	0.003–0.020 (22)

Abbreviation: TOS, total organic solids.

3.5.3 | Uncertainty analysis

In accordance with the guidance provided in the EFSA opinion related to uncertainties in dietary exposure assessment (EFSA, 2006), the following sources of uncertainties have been considered and are summarised in Table 4.

TABLE 4 Qualitative evaluation of the influence of uncertainties on the dietary exposure estimate.

Sources of uncertainties	Direction of impact
Model input data	
Consumption data: different methodologies/representativeness/underreporting/misreporting/no portion size standard	+/–
Use of data from food consumption surveys of a few days to estimate long-term (chronic) exposure for high percentiles (95th percentile)	+
Possible national differences in categorisation and classification of food	+/–
Model assumptions and factors	
Exposure to food enzyme–TOS was always calculated based on the recommended maximum use level	+
Selection of broad FoodEx categories for the exposure assessment	+
For yeast processing, although the food enzyme is not used to treat yeast cell wall,[46] the food categories chosen for calculation covers also those containing mannoproteins resulted from the treatment of yeast cell wall	+
To estimate the exposure from the production of flavouring preparations from dairy products, cheese is the only raw material indicated by the applicant,[47] but the calculation included all types of enzyme-modified dairy ingredients	+
Use of recipe fractions in disaggregation FoodEx categories	+/–
Use of technical factors in the exposure model	+/–

Abbreviations: +, uncertainty with potential to cause overestimation of exposure; –, uncertainty with potential to cause underestimation of exposure; TOS, total organic solids.

[46]Technical dossier/ Additional information April 22/Answer to question 10.

[47]Technical dossier/Additional information April 22/Answer to question 7.

The conservative approach applied to estimate the exposure to the food enzyme–TOS, in particular assumptions made on the occurrence and use levels of this specific food enzyme, is likely to have led to an overestimation of the exposure.

3.6 | Margin of exposure

A comparison of the NOAEL (700 mg TOS/kg bw per day) identified from the 90-day rat study with the derived exposure estimates of 0.001–0.290 mg TOS/kg bw per day at the mean and from 0.003–0.973 mg TOS/kg bw per day at the 95th percentile resulted in a margin of exposure of at least 719.

4 | CONCLUSIONS

Based on the data provided and the derived margin of exposure, the Panel concluded that the food enzyme thermolysin produced with the non-genetically modified *A. caldiproteolyticus* strain AE-TP does not give rise to safety concerns under the intended conditions of use.

5 | DOCUMENTATION AS PROVIDED TO EFSA

Application for authorisation of Thermolysin (Protease) from *Geobacillus stearothermophilus* AE-TP in accordance with Regulation (EC) No 1331/2008. February 2015. Submitted by Amano Enzyme Inc.

Additional information. April 2022 and September 2023. Submitted by Amano Enzyme Inc.

ABBREVIATIONS

ANI	average nucleotide identity
AMR	antimicrobial resistance
BUN	blood urea nitrogen
bw	body weight
CAS	Chemical Abstracts Service
CEP	EFSA Panel on Food Contact Materials, Enzymes and Processing Aids
EINECS	European Inventory of Existing Commercial Chemical Substances
EMC	enzyme-modified cheeses
FAO	Food and Agricultural Organization of the United Nations
GLP	Good Laboratory Practice
GMO	genetically modified organism
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kDa	kiloDalton
LoD	limit of detection
NOAEL	no observed adverse effect level
OECD	Organisation for Economic Cooperation and Development
TOS	total organic solids
WGS	whole genome sequencing
WHO	World Health Organization

CONFLICT OF INTEREST

If you wish to access the declaration of interests of any expert contributing to an EFSA scientific assessment, please contact interestmanagement@efsa.europa.eu.

REQUESTOR

European Commission

QUESTION NUMBER

EFSA-Q-2016-00083

PANEL MEMBERS

José Manuel Barat Baviera, Claudia Bolognesi, Andrew Chesson, Pier Sandro Cocconcelli, Riccardo Crebelli, David Michael Gott, Konrad Grob, Claude Lambré, Evgenia Lampi, Marcel Mengelers, Alicja Mortensen, Gilles Rivière, Inger-Lise Steffensen, Christina Tlustos, Henk Van Loveren, Laurence Vernis, and Holger Zorn.

NOTE

The full opinion will be published in accordance with Article 12 of Regulation (EC) No 1331/2008 once the decision on confidentiality will be received from the European Commission.

REFERENCES

EFSA (European Food Safety Authority). (2006). Opinion of the scientific committee related to uncertainties in dietary exposure assessment. *EFSA Journal*, *5*(1), 438. https://doi.org/10.2903/j.efsa.2007.438

EFSA (European Food Safety Authority). (2009). Guidance of the scientific committee on transparency in the scientific aspects of risk assessments carried out by EFSA. Part 2: General principles. *EFSA Journal*, *7*(5), 1051. https://doi.org/10.2903/j.efsa.2009.1051

EFSA (European Food Safety Authority). (2011). Use of the EFSA comprehensive European food consumption database in exposure assessment. *EFSA Journal*, *9*(3), 2097. https://doi.org/10.2903/j.efsa.2011.2097

EFSA CEF Panel (EFSA Panel on Food Contact Material, Enzymes, Flavourings and Processing Aids). (2009). Guidance on the submission of a dossier on food enzymes. *EFSA Journal*, *7*(8), 1305. https://doi.org/10.2903/j.efsa.2009.1305

EFSA CEP Panel (EFSA Panel on Food Contact Materials, Enzymes and Processing Aids). (2019). Statement on the characterisation of microorganisms used for the production of food enzymes. *EFSA Journal*, *17*(6), 5741. https://doi.org/10.2903/j.efsa.2019.5741

EFSA CEP Panel (EFSA Panel on Food Contact Materials, Enzymes and Processing Aids), Lambré, C., Barat Baviera, J. M., Bolognesi, C., Cocconcelli, P. S., Crebelli, R., Gott, D. M., Grob, K., Lampi, E., Mengelers, M., Mortensen, A., Rivière, G., Steffensen, I.-L., Tlustos, C., Van Loveren, H., Vernis, L., Zorn, H., Glandorf, B., Herman, L., … Chesson, A. (2021). Scientific guidance for the submission of dossiers on food enzymes. *EFSA Journal*, *19*(10), 6851. https://doi.org/10.2903/j.efsa.2021.6851

EFSA CEP Panel (EFSA Panel on Food Contact Materials, Enzymes, Processing Aids), Lambré, C., Barat Baviera, J. M., Bolognesi, C., Cocconcelli, P. S., Crebelli, R., Gott, D. M., Grob, K., Lampi, E., Mengelers, M., Mortensen, A., Rivière, G., Steffensen, I.-L., Tlustos, C., van Loveren, H., Vernis, L., Zorn, H., Roos, Y., Apergi, K., … Chesson, A. (2023). Food manufacturing processes and technical data used in the exposure assessment of food enzymes. *EFSA Journal*, *21*(7), 8094. https://doi.org/10.2903/j.efsa.2023.8094

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms). (2010a). Scientific opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed. *EFSA Journal*, *8*(7), 1700. https://doi.org/10.2903/j.efsa.2010.1700

FAO/WHO (Food and Agriculture Organization of the United Nations/World Health Organization). (2006). General specifications and considerations for enzyme preparations used in food processing in compendium of food additive specifications. 67th meeting. *FAO JECFA Monographs*, *3*, 63–67. https://www.fao.org/3/a-a0675e.pdf

OECD (Organisation for Economic Co-Operation and Development). (2016). OECD Guideline for the testing of chemicals, Section 4 Health effects, Test No. 487: *In vitro* mammalian cell micronucleus test. 29 July 2016. 29 pp. https://doi.org/10.1787/9789264264861-en

OECD (Organisation for Economic Co-Operation and Development). (2018). OECD Guideline for the testing of chemicals, Section 4 Health effects, Test No. 408: Repeated dose 90-day oral toxicity study in rodents. 27 June 2018. 16 pp. https://www.oecd-ilibrary.org/docserver/9789264070707-en.pdf?expires=1660741445&id=id&accname=guest&checksum=96397D93F35C1C4B2EDC61F7C943E708

OECD (Organisation for Economic Co-Operation and Development). (2020). OECD Guideline for the testing of chemicals, Section 4 Health effects, Test No. 471: Bacterial reverse mutation test. 29 June 2020. 11 pp. https://www.oecd-ilibrary.org/docserver/9789264071247-en.pdf?expires=1660741244&id=id&accname=guest&checksum=913CB0A044880B3F24CBF2A547CF413C

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: EFSA CEP Panel (EFSA Panel on Food Contact Materials, Enzymes and Processing Aids), Lambré, C., Barat Baviera, J. M., Bolognesi, C., Cocconcelli, P. S., Crebelli, R., Gott, D. M., Grob, K., Lampi, E., Mengelers, M., Mortensen, A., Rivière, G., Steffensen, I.-L., Tlustos, C., Van Loveren, H., Vernis, L., Zorn, H., Herman, L., Roos, Y., … Chesson, A. (2024). Safety evaluation of the food enzyme thermolysin from the non-genetically modified *Anoxybacillus caldiproteolyticus* strain AE-TP. *EFSA Journal*, *22*(2), e8634. https://doi.org/10.2903/j.efsa.2024.8634

APPENDIX A

Dietary exposure estimates to the food enzyme–TOS in details

Appendix A can be found in the online version of this output (in the 'Supporting information' section). The file contains two sheets, corresponding to two tables.

Table 1: Average and 95th percentile exposure to the food enzyme–TOS per age class, country and survey.

Table 2: Contribution of food categories to the dietary exposure to the food enzyme–TOS per age class, country and survey.

APPENDIX B

Population groups considered for the exposure assessment

Population	Age range	Countries with food consumption surveys covering more than 1 day
Infants	From 12 weeks on up to and including 11 months of age	Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Portugal, Slovenia, Spain
Toddlers	From 12 months up to and including 35 months of age	Belgium, Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Hungary, Italy, Latvia, Netherlands, Portugal, Republic of North Macedonia[a], Serbia[a], Slovenia, Spain
Children	From 36 months up to and including 9 years of age	Austria, Belgium, Bulgaria, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, Netherlands, Portugal, Republic of North Macedonia[a], Serbia[a], Spain, Sweden
Adolescents	From 10 years up to and including 17 years of age	Austria, Belgium, Bosnia and Herzegovina[a], Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, Montenegro[a], Netherlands, Portugal, Romania, Serbia[a], Slovenia, Spain, Sweden
Adults	From 18 years up to and including 64 years of age	Austria, Belgium, Bosnia and Herzegovina[a], Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Montenegro[a], Netherlands, Portugal, Romania, Serbia[a], Slovenia, Spain, Sweden
The elderly[b]	From 65 years of age and older	Austria, Belgium, Cyprus, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Montenegro[a], Netherlands, Portugal, Romania, Serbia[a], Slovenia, Spain, Sweden

[a]Consumption data from these pre-accession countries are not reported in Table 3 of this opinion, however, they are included in Appendix B for testing purpose.

[b]The terms 'children' and 'the elderly' correspond, respectively, to 'other children' and the merge of 'elderly' and 'very elderly' in the Guidance of EFSA on the 'Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment' (EFSA, 2011).